From DEPARTMENT OF LABORATORY MEDICINE
Karolinska Institutet, Stockholm, Sweden

SYNDECAN-1 INSIGHTS IN MESOTHELIOMA

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Karolinska Institutet

Stockholm 2017

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Cover photo evokes syndecan-1 shedding and cancer.
Printed by E-print AB

ISBN 978-91-7676-823-5

SYNDECAN-1 INSIGHTS IN MESOTHELIOMA

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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'' In every job that must be done, there is an element of fun

You find the fun and snap! The job's a game!''

Mary Poppins

To my parents

ABSTRACT

Syndecan-1 is a cell surface heparan sulfate proteoglycan expressing on epithelial cells. Heparan sulfate (HS) chains on syndecan-1 constitute growth factor attachment sites and facilitate growth factors to bind their respective receptors. This binding property of HS chains allows syndecan-1 to be involved in various cellular processes. The fine structure of HS defines the binding properties of these chains. Sulfatase-1 is one of the enzymes that regulates sulfation pattern of HS chains. On the cell membrane syndecan-1 can get shed and the soluble proteoglycan can compete with cell surface-bound and might have counteracting roles.

Malignant mesothelioma (MM) is highly aggressive tumor of mesothelial cells lining the serosal cavities. Presence of syndecan-1 on the cell surface of MM is associated with favorable prognosis, whereas the decrease of syndecan-1 deteriorates the prognosis. With this thesis work, we aimed to disclose syndecan-1 roles and the underlying mechanisms by which syndecan-1 affect the behavior of malignant mesothelioma.

We focused on genes and pathways modulated by syndecan-1 overexpression and silencing in a mesothelioma cell line (paper I), and we found out that TGF-β, EGF, VEGF and ERK/MAPK pathways were affected in both settings. Syndecan-1 silencing enriched cell cycle pathways and syndecan-1 overexpression had the opposite effects. Syndecan-1 overexpression affected gene expression involved in angiogenesis, adhesion, proliferation, cell cycle, migration, interleukins, extracellular matrix proteins and HS modifying enzymes.

Among the HS modifying enzymes affected by syndecan-1 overexpression, sulfatase-1 gene was highly downregulated (paper III). HS content was decreased but overall sulfation was increased by syndecan-1 overexpression. Studying downstream signaling molecules showed that syndecan-1 affects PI3K and MAPK signaling pathways in mesothelioma, which leads to cell cycle arrest at G1.

Syndecan-1 level was evaluated in two cohorts of patients both in pleural effusions and sera (paper II). Syndecan-1 was elevated in malignant effusions than benign conditions and could predict malignant disease. In addition, patients with higher levels of syndecan-1 in pleural effusion had shorter survival compared to the patients with lower syndecan-1 levels. However these effects were not observed with syndecan-1 levels in serum.

Syndecan-1 overexpression on mesothelioma cells inhibited endothelial cell proliferation, migration and tube formation (paper IV). Endothelial cell tube formation was reverted by MMP7 silencing, which is one of the important sheddases of syndecan-1. Co-cultured HUVEC and mesothelioma cells showed less nuclear Yes-associated protein (YAP) expression, which is associated with less migration. The angiogenesis inhibitory effects of syndecan-1 overexpressing cells were conducted by both pro- and anti-angiogenic factors comprising Angiopoietin-1, FGF-4, HGF, NRG1-β1, TSP-1, TIMP-1 and TGF-β1. VEGF levels in pleural effusions from mesothelioma patients correlate to soluble syndecan-1 levels and have prognostic value in these patients. Combining shed syndecan-1 and VEGF seemed to be better for prognostic evaluation of mesothelioma patients than these factors alone.

LIST OF SCIENTIFIC PAPERS

I. Szatmári T, Mundt F, **Heidari-Hamedani G**, Zong F, Ferolla E, Alexeyenko A, Hjerpe A, Dobra K.
Novel genes and pathways modulated by syndecan-1: implications for the proliferation and cell-cycle regulation of malignant mesothelioma cells. *PLoS One. 2012;7(10):e48091.*

II. Mundt F, **Heidari-Hamedani G**, Nilsonne G, Metintas M, Hjerpe A, Dobra K.
Diagnostic and prognostic value of soluble syndecan-1 in pleural malignancies. *Biomed Res Int. 2014;2014:419853.*

III. **Heidari-Hamedani G**, Vivès RR, Seffouh A, Afratis NA, Oosterhof A, van Kuppevelt TH, Karamanos NK, Metintas M, Hjerpe A, Dobra K, Szatmári T.
Syndecan-1 alters heparan sulfate composition and signaling pathways in malignant mesothelioma. *Cell Signal. 2015 Oct;27(10):2054-67.*

IV. **Heidari-Hamedani G**, Javadi J, Schmalzl A, Szatmári T, Metintas M, Aspenström P, Hjerpe A and Dobra K.
Syndecan-1 overexpressing mesothelioma cells inhibit proliferation, migration and tube formation of endothelial cells by synchronized action of angiogenesis related factors. *Manuscript.*

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LIST OF ABBREVIATIONS

CDK	Cyclin-dependent kinase
CK	Cytokeratin
CS	Chondroitin sulfate
ECM	Extracellular matrix
EGF	Epidermal growth factor
EMA	Epithelial membrane antigen
ERK	Extracellular signal–regulated kinases
FGF	Fibroblast growth factor
FISH	Fluorescence in situ hybridization
GAG	Glycosaminoglycan
GalNAc	N-acetylgalactosamine
GlcNAc	N-acetylglucosamine
GlcUA	Glucuronic acid
GSEA	Gene set enrichment analysis
HA	Hyaluronic acid
HBME1	Hector battifora mesothelial 1
HGF	Hepatocyte growth factor
HS	Heparan sulfate
HUVEC	Human umbilical vein endothelial cells
IdoUA	Iduronic acid
IL	Interleukin
IPA	Ingenuity pathway analysis
MAPK	Mitogen-activated protein kinase
MM	Malignant mesothelioma
MMP	Matrix metalloproteinase
MPM	Malignant pleural mesothelioma
NDST	N-deacetylase N-sulfotransferase
NEA	Network enrichment analysis
OPN	Osteopontin

PDGF	Platelet-derived growth factor
PI	Propidium Iodide
PI3K	Phosphatidylinositol-3-kinase
ROC	Receiver operating characteristic
SULF	Sulfatase
TGFß	Transforming growth factor ß
TKI	Tyrosine kinase inhibitor
VEGF	Vascular endothelial growth factor
WST-1	Water soluble tetrazolium salt-1
WT-1	Wilms tumor-1
YAP	Yes associated protein

1 BACKGROUND

1.1 MALIGNANT MESOTHELIOMA

1.1.1 Types, aetiology and epidemiology

Malignant mesothelioma (MM) is a tumor arising from mesothelial cells that cover the lungs (pleura), heart (pericardium), abdomen (peritoneum) and a mesothelial remain is seen in the testis capsule (tunica vaginalis). The most frequently occurring type is malignant pleural mesothelioma (MPM) which accounts for nearly 70% of all mesothelioma cases [1, 2], and it is the focus of this thesis.

The main cause of MM is asbestos exposure. Asbestos is a carcinogenic mineral fiber that has been widespreadly used in the past, and has been prohibited nowadays in western countries, while its use in countries like China, India and Brazil is higher than ever. However, due to the long latency of mesothelioma which is between 20-40 years [1, 3] and differences in the prohibition rules of asbestos in various countries, mesothelioma incidence remains an increasing problem worldwide [4]. Although 90% of mesothelioma cases are associated with asbestos exposure, 10% are thought to develop mesothelioma due to genetic predispositions or other asbestos-like materials such as erionite [5], which are other reasons for constant prevalence of this disease [6]. In Cappadocia region of Turkey, erionite containing rocks have been used in constructing and painting houses. Erionite tumorigenesis has been shown to be the strong cause for high incidence of mesotheliomas in this area [5].

The incidence of mesothelioma in Western Australia is the highest in the world [7] mainly due to the Wittenoom mine. In Europe the highest incidence is found in Great Britain, the Netherlands, Malta and Belgium. Sweden is among the countries with intermediate incidence rate, with 100 annual cases of mesothelioma [8].

1.1.2 Pleural effusions

The pleura consist of a thin flat monolayer of mesothelial cells resting on a basement membrane with fibroblast-like progenitor cells underneath. Although mesothelial cells arise from the mesoderm and have NCAM (neural cell adhesion molecule) for attachment, i.e., classifying these cells as mesenchymal, these mesenchymal cells mimic epithelial cells in terms of covering a surface motivating the term “mesothelium” [9, 10]. The pleura cover the lungs and chest wall and consist of two layers. The space between these two layers of pleura forms the pleural cavity, which contains normally only a minute volume of fluid and low cellular content, including macrophages, mesothelial cells, and lymphocytes (Figure 1) [11]. This fluid aids lungs expansion during breathing by achieving low friction, and it contains growth factors, cytokines and chemokines such as transforming growth factor ß (TGFß), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), IL1, IL15, IL16, IL18, etc. These components maintain the cellular integrity and help leukocyte infiltration in case of inflammatory response [12]. Pleural fluid is constantly secreted into the pleural cavity from the intercostal arteries and it is resorbed by lymphatic stomata on the parietal pleural surface [13]. In normal conditions the volume of pleural fluid does not exceed more than few milliliters (0.1–0.2 mL/kg), but in pathological

conditions of pleura it is dramatically increased, either due to increased production of pleural fluid and/or inhibited reabsorption. Increased formation of this fluid is either due to altered hydrostatic blood pressure or microvascular permeability, while infiltration of malignant cells may block the lymphatic drainage [14].

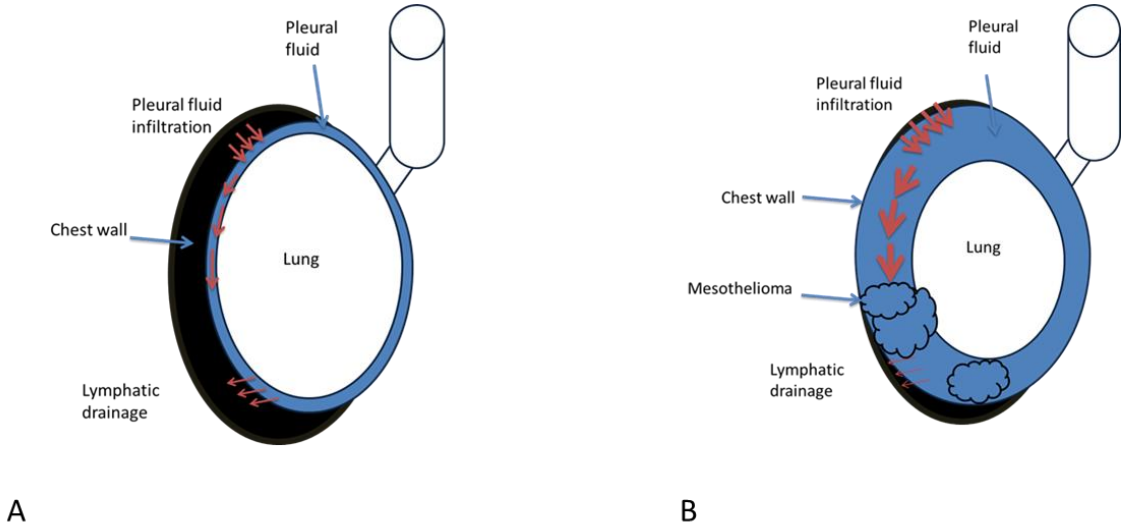

Figure 1. Oversimplified depiction of pleural cavity made from the space between mesothelial lining covering each lung. A) Healthy condition, B) Mesothelioma.

Pleural effusions are often the results of circulatory congestion (transudate), or inflammatory lung disease (exudate). It could also be the result of metastases from other tumors such as metastatic adenocarcinomas from the lung (36%), breast (25%), malignant lymphomas (10%) and ovary and gastric adenocarcinoma (5%) [15]. Malignant pleural effusions can also be due to primary tumor of pleura which is MM, however with less frequent occurrence than secondary cancers. Since the subject of this thesis is MPM, the further focus will be on this kind of cancer.

1.1.3 Diagnosis of malignant pleural mesothelioma

MPM is one of the most aggressive malignant tumors with a long latency from asbestos exposure to disease manifestation. Patients have a short survival time between 9 to 12 months after diagnosis [4]. Diagnosis of mesothelioma is challenging particularly differentiating MPM from benign pleural effusions or other malignancies [16]. Disease symptoms have often slow onset and include dyspnea, weight loss, chest pain, cough, and accumulation of pleural fluid (effusions), which impede the breathing and is one of the most important symptoms. The diagnosis of pleural malignancies and the differentiation between metastatic pleural disease and MPM require morphological evaluations [17, 18].

1.1.3.1 Histology and Cytology

Histologically there are three distinct phenotypes in MPM. One is known as epithelioid, in which cells show polygonal or cuboidal morphology. Epithelioid subtype is the most common type of mesothelioma (around 60% of all mesotheliomas). The second subtype is the sarcomatoid phenotype with fibroblast-like cells (around 10% of all mesothelioma), while

the third phenotype is biphasic with a mixture of both epithelioid and sarcomatoid cells (around 30%). The sarcomatoid phenotype has a worse prognosis than epithelioid phenotype, with higher rate of resistance to therapy [19-23]. The histological subtype of mesothelioma is associated with survival. The epithelioid phenotype is associated with longer survival compared with sarcomatoid and biphasic types [24]. This difference in therapy response and prognosis can be related to the differences in gene expression profile between the two phenotypes [25-27]. According to previous recommendations, diagnosis of MPM is based on histology [28], while recent recommendation from International Mesothelioma Interest Group is that cytological diagnosis of epithelioid mesothelioma is possible with advantages of using pleural effusions as less invasive method than biopsy and faster confirmation of diagnosis [16, 29]. One disadvantage of this method is that the sarcomatoid mesotheliomas will not be perceived as this phenotype usually does not shed malignant cells into the pleura.

Histological diagnosis is based on the hematoxylin-eosin staining of tissues, also using a panel of immunohistochemical markers. Such a panel should contain positive markers for mesothelioma and excluding markers. The challenge in epithelioid morphology is to differentiate from metastatic adenocarcinomas as well as from reactive mesothelial proliferations. Recognition of the sarcomatoid or mixed phenotypes can be critical in clinical decision making. Differential diagnosis of sarcomatoid mesothelioma and other spindle cell neoplasm and also differential diagnosis of biphasic mesothelioma and other biphasic tumors such as synovial sarcoma are challenging [30].

Cytological diagnosis can be performed based on the effusion that is obtained by thoracentesis to reduce patients' symptoms by facilitating their breathing. The most important cytological features of MM include high cellularity, with numerous cell groups of varying size. The nuclear atypia may be substantial, but is often blend and difficult to distinguish from a reactive mesothelial condition. The cell groups may contain acidophilic extracellular matrix cores, and the cytological specimens sometimes show an acidophilic granular background, correlating to hyaluronan production of the tumor [29].

1.1.3.2 Adjuvant techniques

The diagnosis will be performed using adjuvant techniques such as fluorescence in situ hybridization (FISH), immunocytochemistry or immunohistochemistry, electron microscopy and biomarkers.

FISH can be used to demonstrate chromosomal abnormalities such as aneuploidy, which indicates malignancy [31]. Therefore, the main use of FISH analysis is to distinguish malignant from reactive cells. A commonly used commercial kit (Urovysion, Abbot) adopted also for pleural effusions uses three fluorescence probes to centromeric sequences on chromosomes 3, 7 and 17 showing chromosomal gains or losses and a fourth probe that labels the 9p21 band, containing the tumor suppressor cyclin-dependent kinase inhibitor gene p16, p16INK gene (*CDKN2A*). A homozygous deletion of the 9p21 band is a common finding in MM [31].

Immunocytochemistry is a well-established method to obtain diagnosis, however the challenge of determining reactive cells exists using this method. Although some reports

suggest that strong membranous staining with antibody against epithelial membrane antigen (EMA) from the E29 clone is a good marker for distinguishing mesothelioma from reactive mesothelial hyperplasia [32, 33]. Simultaneous desmin and calretinin positivity is also indicative for a reactive condition.

Immunological markers of mesothelial lineage are calretinin, mesothelin, wilms tumor-1 (WT1), HBME-1, CK 5/6, thrombomodulin and D2-40 (podoplanin). Calretinin, WT-1 and D2-40 have so far the greatest specificity for mesothelioma. In order to differentiate lung adenocarcinoma from mesothelioma, thyroid transcription factor-1 (TTF-1) and/or napsin A could be used [23]. In addition, estrogen receptor alpha (ER) and mammaglobin (MG) are good differentiating markers for breast adenocarcinoma from mesothelioma [34].

Electron microscopy is another useful tool in mesothelioma diagnosis. This method is especially useful when differentiating between mesothelioma and other metastatic malignancies. The ultra-structure of mesothelial cell include long thin microvilli on cell surface, abundant intermediate filaments, and prominent accumulations of intracytoplasmic glycogen and the formation of neolumina [35].

1.1.3.3 Soluble Biomarkers

Biomarkers can be helpful in establishing the diagnosis earlier, assessing prognosis and predicting theraputical responses in patients. Yet, diagnostic, prognostic or predictive biomarkers are not widely established in clinical practice, although there are several mesothelioma biomarkers described. Depending on whether these biomarkers are measured in pleural effusions or serum, and on the analytical method used in the laboratories, biomarkers demonstrate varying specificities and sensitivities.

Hyaluronic acid (HA) or hyaluronan is an extracellular polysaccharide in connective tissues. It has been reported that effusion levels of HA in MPM patients could be a diagnostic marker [36, 37], with sensitivity comparable to mesothelin [38], although with better performance in receiver operating characteristic (ROC) plot analysis. Moreover, a two-step model has been proposed with high specificity using hyaluronan and N-ERC/mesothelin in effusions to predict mesothelioma [39].

To date, the cell surface glycoprotein mesothelin, when measured in serum or pleural effusion, is a good adjuvant diagnostic marker, although with less specificity than HA. The term soluble mesothelin related protein (SMRP) is given to isoforms of mesothelin which can be found in blood [40]. Several studies have shown that SMRP level in serum or pleural effusions of patients with advanced stage epithelioid and biphasic MPM was higher compared to early stage, and decreased SMRP level has been reported as an indicator of tumor shrinkage after therapy [34]. Data suggest that mesothelin could be used as a prognostic marker indicating poor survival [41-43].

Osteopontin (OPN) is highly phosphorylated matricellular protein involved in the formation of teeth and bone matrix and type I immune responses. OPN has been suggested as another marker for mesothelioma diagnosis with effective diagnostic accuracy in serum and plasma [44].

MicroRNAs (miRNAs) are short (19-25 nucleotides) non-coding RNAs, which are involved in various cellular processes by affecting post-transcriptional regulation of genes. In MPM, as other tumors, microRNAs are dysregulated [45]. Several micoRNAs are identified so far in MM cell lines, tumors and patients' blood, such as miR-29c*, by which increased expression predicted a more favorable prognosis [46]. In addition, in biological samples such as plasma, increased levels of miR-29c*, miR-92a and miR-625-3p are suggested as tumor markers for MM [47]. In general, miRNAs in the MM tumors and peripheral blood of patients seem to serve as potential biomarkers and/or therapeutic targets.

1.1.4 Molecular pathogenesis of malignant mesothelioma

The pathogenesis of MM is known to be multifactorial. Although around 90% of mesothelioma patients have been exposed to asbestos, other factors such as familial predispositions, radiotherapy, Simian virus 40 infection, and genetic and environmental factors potentiate the mesothelioma development which details are beyond the scope of this thesis.

There are four proposed mechanisms by which asbestos is thought to damage pleura. First, making a physical damage by deep penetration of asbestos fibers when they are inhaled and irritating the pleura [48]. Second, asbestos fibers might interfere with the mitotic spindle of cells and consequently mitosis, which leads to aneuploidy and other chromosomal damage [49]. Third, asbestos produces iron-related reactive oxygen species which damage DNA [50]. Fourth, asbestos phosphorylates two kinases of mitogen-activated protein (MAP) kinases and extracellular signal–regulated kinases (ERK) 1 and 2. The higher activity of these kinases enhances the expression of early-response proto-oncogenes that encode members of the Fos–Jun and activator protein 1 families [51, 52].

At the molecular level, growing evidence shows accumulation of a broad spectrum of genetic changes that lead to cancer. Some of the most common abnormalities are chromosomal loss of the short arm (p) of chromosomes 1, 3, and 9, and loss of the genetic material from the long arm (q) of chromosomes 6, 13, and 15. In addition anomaly in regulatory genes p16 and p14 which are expressed at the 9p21 locus (*p16INK4a, p14ARF*), chromosome 22 (*NF2*), and loss of 3p21 is the result of mutation of the *BAP1* gene (BRCA 1-associated protein 1) [53].

The cyclin-dependent kinase inhibitor 2A (*CDKN2A*)/ alternative reading frame (ARF) gene is one of the most frequently inactivated tumor suppressor genes in MM. *CDKN2A* locus encodes p16INK4a, which inhibits the progression of G1 to S phase of cell cycle via the cyclin-dependent kinase 4/cyclin D- retinoblastoma protein [54]. *ARF* encodes p14ARF, which stabilizes p53 and retinoblastoma protein, thus causing G1 and G2 arrest in cell cycle [55, 56].

NF2 encodes a protein called merlin with tumor suppressor properties. Merlin affects cell proliferation and growth via regulating the Hippo and mammalian target of rapamycin (mTOR) pathways [57].

The importance of familial genetic susceptibility is pronounced in *BAP1* mutation, where families with this mutation developed various kinds of cancer, including mesothelioma [58-

60]. *BAP1* encodes deubiquitinating enzymes which are involved in histone modification and therefore affecting global gene expression profiling, acting as tumor suppressors [61].

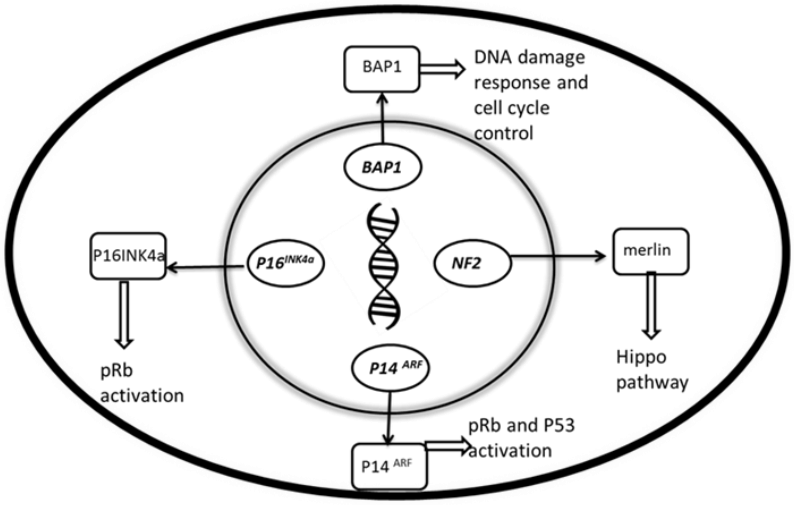

Figure 2. Summary of genes and proteins which are involved in MM development

1.1.5 Role of intracellular signaling in malignant pleural mesothelioma

The "molecular pathology" concept refers to signaling molecules and transcription factors from different pathways which are involved in tumor pathology. MPM exposure to asbestos fibers initiates multiple cell signaling pathway abnormalities, which consequently lead to cancer [53].

Several enhanced receptor activations due to growth factor binding, or inactivating mutations in the *NF2* gene cause activation of intracellular signal transduction pathways. Bodies of data demonstrate aberrant activation of phosphatidylinositol-3-kinase and protein kinase B (PI3K/AKT), ras/raf/MEK/MAPK pathways [53, 62], Hippo signaling cascade [63] and Hedgehog signaling [64]. These changes will ultimately lead to excessive proliferation, angiogenesis, invasion, metastasis, as well as resistance to therapy.

Epidermal growth factor (EGF) and its receptor (EGFR) have been reported to be involved in mesothelioma development and progression by receptor auto-phosphorylation in mesothelial cells after asbestos exposure leading to MAPK cascade activation and inducing proto-oncogenes c-fos and c-jun and initiating the carcinogenesis process [65].

Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) are two prominent angiogenic factors. It has been reported that high VEGF levels are observed in pleural effusions from mesothelioma patients compared to non-malignant group and there is inverse correlation between VEGF levels and mesothelioma patient's survival [66]. High levels of bFGF in mesothelioma pleural effusion was found to be correlated to shorter survival, however the levels were lower in mesothelioma compared to non-malignant group [67].

One of the important growth factors in mesothelioma is transforming growth factor-α (TGF-α), which binds to EGFR with high affinity and it inserts its effects through activation of tyrosine kinase activity of this receptor [68]. Transforming growth factor β1 (TGF-β1) affects VEGF production and together they are involved in pleural effusion formation and mesothelioma cell growth [63, 69].

Platelet-derived growth factor (PDGF) could have both autocrine/paracrine growth factor roles for mesothelioma cells. mRNAs from PDGF A- and B- chains are higher in mesothelioma compared to normal mesothelial cells [53, 70].

High levels of hepatocyte growth factor (HGF) and keratinocyte growth factor (KGF), have been detected in pleural effusions of rats receiving asbestos intra-tracheally [71]. In mesothelioma patients, high levels of HGF in serum compared to healthy individuals [72] and immunoreactivity of tumor sections for HGF and its receptor Met have been demonstrated [73]. High signaling activities of AKT and ERK1/2 have been observed due to increased level of HGF that resulted in increased migration, cell division and invasiveness of mesothelioma cells [74].

1.1.6 Angiogenesis in malignant pleural mesothelioma

During carcinogenesis, when tumor is less than 0.5 to 1 mm in diameter, there is a balance between pro- and anti-angiogenic factors. This results in the so called ‘’angiogenic switch’’ to be off. However, when this switch gets on and the balance favors pro-angiogenic factors, tumor starts to vascularize, and metastatic growth initiates [75].

VEGF is the most prominent angiogenic factor with roles in vessel hyper-permeability and carcinogenesis. High expression of VEGF has been found in epithelioid and biphasic mesothelioma [53, 76]. Several studies show that vessel permeability and microvascular density positively correlate with VEGF levels in malignant pleural effusions. In addition, increased microvessel density is suggested to be independent predictor of poor prognosis in mesothelioma [77, 78]. Moreover, VEGF receptors (VEGFR-1, VEGFR-2 and VEGFR-3) are highly expressed in MPM [79, 80].

Bevacizumab is an antibody which neutralizes VEGF and has shown activity in various cancer types such as metastatic colorectal cancer, metastatic non-squamous non-small cell lung cancer and metastatic breast cancer. The use of bevacizumab in combination with pemetrexed plus cisplatin significantly increased survival in MPM with manageable side effects [81-83].

Since other tyrosin kinase receptors such as FGF, HGF and PDGF are also involved in mesothelioma pathogenesis (mentioned earlier in 1.1.5), multi-targeted tyrosine kinase inhibitors (TKIs), are other options for treatment, which are still evaluated in clinical trials. However, to date no significant positive results have been reported [81]. An example is using Axitinib (VEGF and PDGF receptors inhibitor) in combination with cisplatin and pemetrexed in MPM patients, where Axitinib could reduce angiogenesis; however, there was no positive clinical outcome [84].

1.1.7 Treatment of malignant mesothelioma

Mesothelioma is often resistant to chemotherapy. The standard treatment is cisplatin and pemetrexed combination which increases patient's survival up to 2.8 months compared to single treatment with cisplatin [85]. On the other hand sarcomatoid and biphasic MPM demonstrate poor response to standard treatment and associate with worse prognoses [86]. Several other regimens have been studied as second-line therapy but results are not convincing yet. Since NF2 gene inactivation causes deregulation in intracellular signaling and affecting FAK, ERK, HER1 and 2 and mTOR pathways, an mTOR inhibitor, was tested in MPM and demonstrated median progression free survival of 2.8 months [87]. Several TKIs are in trials for MPM, such as sorafenib (TKI against VEGFR isoforms, PDGFR-α and β, EGFR), sunitinib (TKI against VEGFR, PDGFR, c-Kit), gefitinib and erlotinib (TKI against EGFR). No significant response is seen in MPM treatment using these agents yet [86].

Radiotherapy is mostly used to treat pain in MPM [88, 89]. Since mesothelioma is a diffuse disease often engaging large areas or the entire pleura, radiotherapy should be excessive and cover the whole hemithorax [90]. In a study of postoperative radiotherapy, there was no significant difference in median survival between those who received radiotherapy after surgery and chemotherapy and the group who did not receive radiotherapy [91].

Surgery (pleuropneumonectomy) might be an option especially at early stages of disease when mesothelioma is still localized and metastasis has not occurred yet. However, guidelines are different in the world and surgery is not performed in all countries, including Sweden. Surgery could be performed either by removing the whole affected pleura and lungs and sometimes diaphragm (extrapleural pneumonectomy (EPP)), or less aggressively pleurectomy/decortication or resection (P/D) of tumor tissue in pleura, lung and chest wall [92, 93]. The mortality rate after EPP is relatively high, (32% in the past and recently with surgical techniques development is reduced to 4%). Mortality rate by P/D is 1.5-5.4%. In general, if disease is still progressive after chemotherapy, surgery is not recommended [89].

1.2 SYNDECAN-1 BIOLOGY

1.2.1 Proteoglycans

Proteoglycans (PGs) are abundant molecules on the cell surface, in the extracellular matrix and intracellular compartments. These molecules are composed of a core protein bearing covalently attached glycosaminoglycan (GAG) chains. Based on the disaccharide structures of GAG chains which will be explained later, there are six types of GAGs: the galactosaminoglycans chondroitin sulfate (CS), and dermatan sulfate (DS), the glucosaminoglycans heparan sulfate (HS), heparin, keratan sulfate (KS), and Hyaluronan (HA) [94, 95].

Except for hyaluronan (HA), which is freely present in the extracellular matrix, other GAG chains are parts of PGs [94, 96]. PGs serve their biological functions through both GAG chains and core proteins. These functions are through binding the positively charged effector proteins such as growth factors, cytokines and chemokines to GAG chains, or complex formations through the core protein with other proteins as integrins and modulating their intracellular signaling [97]. Therefore, these complex molecules are involved in wide range of cell processes from proliferation, angiogenesis, migration, adhesion and motility to apoptosis, both in physiological and pathological conditions such as organogenesis, and tumor progression.

1.2.2 Syndecans

There are two major cell surface PG families: syndecans, which are transmembrane PGs, and glypicans, which are covalently attached to the membrane by a glycophosphatidylinositol (GPI) lipid anchor [96]. The mammalian syndecans are a family of four members: syndecan-1-4. Syndecan-1/CD138 is mostly expressed by epithelial cells and plasma cells, syndecan-2/fibroglycan is found on mesenchymal cells, syndecan-3/N-syndecan is expressed on neural crest-derived cells and cartilage, while syndecan-4/amphiglycan is expressed ubiquitously, but at lower levels than other syndecans. [98]. However, expression of syndecan family members might be affected by other members due to compensatory mechanisms [99].

1.2.3 Syndecans' structure

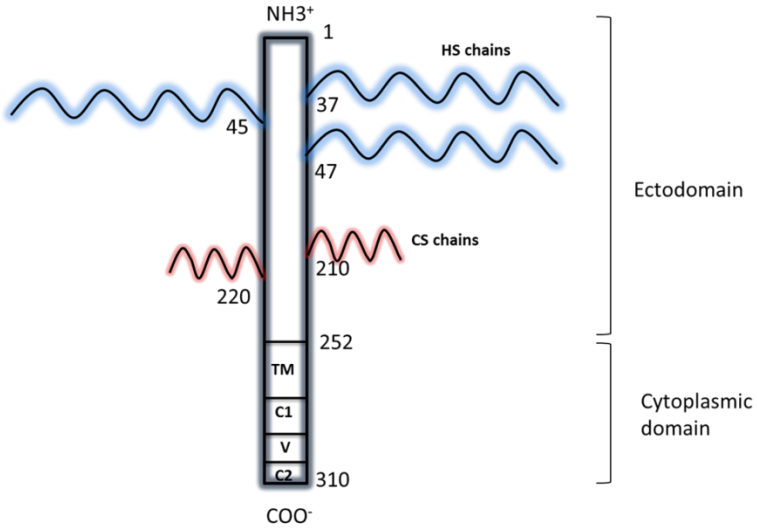

Figure 3. Syndecan-1 is a prototype in syndecan family. TM is transmembrane domain, C1 and C2 are conserved regions 1 and 2, V is the variable region in syndecan family. Heparan sulfate (HS) and chondroitin sulfate (CS) chains are attached to the ectodomain. For heparan sulfate attachment, there are three highly conserved serine-glycine sites amino acids 37,45 and 47 close to the N terminal of the core protein and chondroitin sulfates are attached to two highly conserved serine-glycine sites amino acids 210 and 220 [100].

1.2.3.1 Core protein

Syndecans are single passed type I transmembrane PGs, with a short C-terminus cytoplasmic domain, a transmembrane domain and an N-terminal ectodomain. The whole core protein has a molecular weight between 20-40 kDa.

Within the cytoplasmic domain there are two conserved C1 and C2 regions which have flanked a region that varies between the four members of syndecan (V region). C1 region interacts with PDZ-containing intracellular proteins such as ezrin by which syndecan-2 can interact with actin [101]. This region is involved in endocytosis, exosome biogenesis, nuclear localization [102]. This domain also contains the consensus sequence: RMKKK, which has been shown to be nuclear localization signal for syndecan-1 [103]. The C2 region can interact with PDZ proteins such as syntenin, synectin and can also act as phosphorylation site. V region interacts with actin and fascin, and in this way, affect the cell spreading. In syndecan-2 and -4 it has been shown that through V region, a ternary signaling complex with phosphatidylinositol 4,5-bisphosphate and protein kinase Cα is formed and leads to formation of microfilament bundles and focal adhesions [104-106]

The transmembrane domain of syndecans contains GXXXG motif that is responsible for dimers formation. The biological consequence of this dimerization is still unknown, however GXXXG motif has been linked to stability and activation of signaling complexes in integrin and erbB receptors [106, 107]. Moreover, to what extent syndecans form hetero-dimers in the living cell is still unclear. Syndecans can dimerize with different affinities, for instance syndecan-1 transmembrane domain dimerizes weakly and the syndecan-2 transmembrane domain dimerizes very strongly [107].

The ectodomain of syndecans varies among syndecans, however it has conserved sites for GAG chains attachment. Syndecan-1 has five attachment sites for GAG chains: three are close to the N-terminus and two are near the plasma membrane. These GAG chains are mainly HS chains attached to serine residues of a serine–glycine motif. In addition to HS chains, syndecan-1 and -3 may also have CS chains. HS can be found close to the N-terminus, however CS chains are placed close to the cell membrane [102, 108].

Ectodomain is crucial in interacting with a wide range of ligands in extracellular matrix, such as growth factors, cytokines, chemokines, collagens and proteinases. These interactions are mediated through HS chains. The extracellular domain can get shed through the specific enzymes that cleave the protein core at specific locations, which will be discussed later. The shedding is constant, but it could increase in some pathological conditions such as inflammation, cancer progression, metastasis or wound healing [95]. The spatial location of HS chains close to N-terminus seems to facilitate these interactions with biological effectors. In addition, the presence of CS chain near the plasma membrane could affect syndecan-1 clustering, association with other transmembrane receptors and change the susceptibility to cleavage proteases [102].

1.2.3.2 GAG chains

Sulfated GAG chains are polymers composed of repeating disaccharides, which form long highly negatively charged polysaccharides. These disaccharides might be N-acetylated hexosamines (N-acetyl-D-galactosamine; GalNAc or N-acetyl-D-glucosamine; GlcNAc) bound to a D-/L-hexuronic acid (D-glucuronic acid; GlcUA or L-iduronic acid; IdoUA) or in one case D-galactose (Gal).

The complex synthesis of the sulfated GAG chains occurs in the Golgi apparatus. Both HS and CS/DS chain synthesis initiate with the formation of a tetrasaccharide linkage to the hydroxyl group of serine residues on the ectodomain. This linkage consists of xylose–galactose–galactose–glucuronic acid residues, which will be followed later by the repeating disaccharides. These disaccharides are glucuronic acid (GlcUA)–N-acetylglucosamine (GlcNAc) in HS or GlcUA–N-acetylgalactosamine (GalNAc) in CS chains which will be added to the linkage by EXTL 1-3 transferases. GlcUA residues on CS can then be epimerized into L-iduronic acid (IdoUA) which can transform this GAG to DS [109]. How exactly the balance between HS and CS/DS biosynthesis is regulated at this step, remains to be studied *in vivo*.

The chain elongation termination mechanism is still not clear to date. Once the HS chain is around 50–150 disaccharides, these chains undergo several enzymatic modifications. These modifications include deacetylations, sulfations and epimerization. Chain modifications do not occur uniformly and not on all the synthesized chains, so some parts of HS chains for instance can be more sulfated than other parts. These result in highly diverse chains at the end of process [108, 110].

In HS the first modification enzyme is the N-deacetylase/N-sulfotransferase (NDST1-2) that replaces N-acetyl group of GlcNAc with a sulfate group resulting in N-sulfoglucosamin (GlcNS). Then the next modification is C5-epimerization of GlcUA residues adjacent to GlcNS residues to IdoUA units.

These modified disaccharides will then undergo a series of O-sulfations. An iduronosyl 2-O-sulfotransferase (2-OST), followed by a glucosaminyl 6-O-sulfotransferase (6-OST1-3), and glucosaminyl 3-O-sulfotransferase (3-OST1-7) will modify the chains. The source of sulfate for these reactions is 3′-phosphoadenosine 5′-phosphosulfate (PAPS) which is synthesized from ATP and SO3. There are multiple isoforms for sulfotransferases, which some are tissue specific and could have different substrate specificities. For instance, although 6-O-sulfotranferases can act on both GlcNAc and GlcNS [111], it has been reported that 6-O sulfation preferentially occurs on GlcNS which is flanked by 2-O-sulfated IdoA [112]. The biosynthesis machinery leads to extensive polysaccharide structural heterogeneity, providing highly specific binding sites for protein ligands. Moreover, HS chains vary among different cell types and tissues, which might explain the regulation of different biological functions [110]. In CS/DS, the chain modification is driven through variable O-sulfations of any free OH group. Thus GalNAc-4, -6, GLcUA-2, or -3 and also IdoUA-2 in DS can be sulfated [109, 113].

After HS chain gets synthesized, there are several mechanisms by which HS chains can be modulated on the cell surface, resulting in even more diversity of chains. One modification is removal of 6-O-sulfate groups from HS on the cell surface. This process is mediated through two endosulfatases SULF1-2 which are secreted to the extracellular space [114]. Another mechanism is by truncation of the whole core protein of the proteoglycan which is known as shedding. Syndecans are for instance shed by matrix metalloproteinases [115]. Moreover, heparanase in the extracellular matrix trims HS, which leads to shorter but potentially more bioactive HS fragments [116].

A study show that HS chains on syndecan-1 and -4 from the same cell type are the same [117], however HS chains from the same core protein on different cells are different [118, 119]. On the other hand, CS chains on syndecan-1 are different from CS chains attached to syndecan-4 in mammary gland cells, while again HS chains from these two core proteins from the same source are the same [120].

1.2.4 Ectodomain shedding of syndecans

Ectodomain shedding of PGs is an important posttranslational modification that regulates pathophysiological processes. All membrane-bound proteoglycans shed regardless of which proteoglycan is expressed in the specific cell. When the truncated part of proteoglycan is released, the amount of cell bound GAG chain decreases, while the extracellular soluble ectodomain can still act as autocrine or paracrine receptor, competitively binding the same ligand as the membrane bound form, and thereby influencing the cell behavior. Syndecan ectodomain undergo proteolytic cleavage caused by groups of enzymes which are referred as sheddases. Shedding is highly regulated and occurs in all mammalian syndecans which bear specific cleavage sites close to cell membrane [95, 121].

The matrix metalloproteinases (MMPs) are syndecan sheddases with broad substrate specificity. Therefore, they are involved in many physiological processes such as development and reproduction and matrix remodeling, and also in pathological conditions as inflammatory disease and cancer progression and invasion. MMPs usually cleave before

hydrophobic residues as Leu, Ile, Met, Phe or Tyr, while cleavage before a charged residue is rarely seen [122].

Matrilysin (MMP-7) [123] and membrane-associated metalloproteinases MT1-MMP and MT3-MMP can cleave syndecan-1 [124]. MMP-2 and MMP-9 can cleave syndecans-1, -2 and -4 [125, 126]. The precise cleavage site can vary, depending on the proteases. These proteolyses take place on amino acids clusters located 6–15 residues from the plasma membrane. MT1-MMP cleavage site on human syndecan-1 is at Gly245–Leu246 [124, 127]. Human syndecan-4 is shed at Lys114–Arg115/Lys192–Val130 and Lys114–Arg115, through serine proteases plasmin and thrombin, respectively [128].

Syndecan-1 shedding is enhanced in physiological and pathological conditions, such as physiological response to growth factors [129], chemokines [130], cellular stress [115], wound healing [131]. Likewise enhanced shedding is seen in sepsis [132], multiple myeloma [133], lung cancer [134] and Hodgkin's lymphoma. Shedding can also be experimentally increased due to trypsin [135], and heparanase [136].

The cleaved syndecan ectodomain can accumulate in the physiological compartments, for example syndecan-1 accumulation in skin [137] during wound healing and in serum of cancer patients.

It has been shown that GAG chains play important roles in modulating the shedding. A reduction of GAG content on syndecan-1 by recombinant human heparanase or bacterial heparinase III increased syndecan-1 shedding [138, 139]. There are several proposed mechanisms for this: 1) HS chains physically block the access of sheddases to their cleavage sites, 2) core protein containing HS chains is more stable in a conformation with less susceptibility to proteolysis, 3) HS chains maintain the syndecan- Rab5 complex [136]. Rab-5 is a small GTPase regulating intracellular trafficking and signaling pathways which is bound to syndecan-1 cytoplasmic domain and can initiate syndecan-1 shedding [140].

1.2.5 Syndecan-1, prototype member of the syndecan family

During embryogenesis syndecan-1 is expressed during epithelial-mesenchymal transitions by mesenchymal cells. Later syndecan-1 expression will be limited to epithelial cells, pre-B cells and plasma cells [141].

Syndecan-1 is mainly expressed on the basolateral surface of epithelial cells [142], and bind to extracellular matrix (ECM) components, such as fibronectin [143], collagens I, III and V [144], and thrombospondin [145]. Moreover, the intracellular domain acts as an anchor and connects to actin cytoskeleton [142]. Therefore, this HSPG received the name 'syndecan' from the Greek word: syndein, which means to bind together, as is able to stabilize the epithelial sheets via connecting the ECM to the intracellular cytoskeleton.

As other members of syndecan family, syndecan-1 can regulate a wide variety of biological activities through HS chains binding to a wide range of bioactive effectors such as vascular endothelial growth factors (VEGFs), transforming growth factor-β (TGF-β), fibroblast growth factors (FGFs), hepatocyte growth factor (HGF), platelet-derived growth factors (PDGFs), and cytokines. These interactions influence the biological functions including

developmental processes, proliferation, apoptosis, wound healing, migration, angiogenesis, blood coagulation, tumor metastasis, infection, and inflammation. All these roles are crucial in development and progression of cancer [98].

Depending on the cell type, the fine structure of GAGs on syndecans can be different. This leads to various ligand affinities, function and activity of syndecan-1 among tissues. For example, syndecan-1 on simple epithelial cells carry more and larger HS and CS chains than syndecan-1 on stratified epithelial cells [146]. In addition, syndecan-1 from NIH-3T3 cells can bind laminin [147], whereas syndecan-1 from a mammary epithelial cell line cannot [148].

Interestingly, mice lacking syndecan-1 are fertile and healthy. However, when these mice were challenged with disease-causing agents or conditions, dramatic pathological phenotypes emerged [149]. This suggests that there is a compensatory mechanism by other syndecans or HSPGs which can be functional even when syndecan-1 is lost during normal development, but this mechanism is not efficient in certain post-developmental processes, such as the pathogenesis of diseases. This implies a crucial role for pathogenic involvement of syndecan-1 [98].

1.2.6 Syndecan-1 in cancer

The epithelial-mesenchymal transition (EMT) occurs during cancer progression. During this process epithelial cells gain mesenchymal cells characteristics, whereby they become more mobile resulting in invasion and metastasis [150]. Transforming growth factor-beta (TGF-β) is shown to be involved with activation of EMT inducing transcription factors [151]. This change is reversible and when cells are settled down to form a metastasis, cells can return to their original epithelial phenotype, this process is named mesenchymal-epithelial transition (MET) [152]. Epithelial cells have adherent junctions and tight cell-cell interactions which are mainly mediated through E-cadherin from one cell adhering to the neighboring cells. The cytoplasmic end of this protein chain is associated with actin filaments [153]. During EMT syndecan-1 and E-cadherin are both lost. Depletion of these two alters morphology and anchorage-dependent growth, causing enhancement of cellular motility [154, 155]. Therefore, syndecan-1 presence is necessary to maintain the epithelial morphology.

As other proteoglycans, syndecan-1 has important roles in tumor biology through altering key processes of tumorigenesis, such as cell proliferation, angiogenesis, apoptosis, etc. [156]. The expression of syndecan-1 is dysregulated in different cancer types such as mesothelioma [157, 158] and carcinomas of the lung [159], prostate [160], pancreas [161], breast [162], ovary [163] and colon [164] and multiple myeloma . However, it is noteworthy that depending on the cancer type, the levels of surface or soluble (shed) syndecan-1 might influence the prognosis. For example, in head and neck [165], lung [159] and colorectal [166] cancers, low syndecan-1 expression in tissue is associated with worse prognosis, while high levels of shed syndecan-1 in serum correlate with a poor prognosis in lung cancer [134] and myeloma [133].

Table 1. Adapted from Szatmári T. et al. 2015 [167]. + demonstrates good prognosis, - stands for poor prognosis and ± shows conflicting data.

Cancer type		Stromal syndecan-1	Cell surface syndecan-1	Soluble syndecan-1
Intrathoracic cancers	Mesothelioma		**+**	**-**
	Lung		**+**	**-**
Skin cancers	Basal cell carcinoma		**+**	
	Squamous cell carcinoma	**+**	**+**	
Head and neck cancers	Head and neck	**-**	**+**	
	Laryngeal, hypopharynx			**-**
	Nasopharyngeal		**-**	
Gastrointestinal cancers	Gastric	**-**	**+**	
	Colorectal	**-**	**±**	
	Hepatocellular		**+**	
	Pancreatic		**-**	
Breast cancer	Breast cancer	**±**	**±**	**-**
Urogenital cancers	Cervical		**+**	
	Ovarian	**-**	**-**	
	Endometrial	**-**	**±**	
	Prostate	**-**	**±**	
	Bladder	**-**	**+**	**-**
	Urothelial	**-**		
Hematological malignancies	Myeloma		**-**	**-**
	Hodgkin's lymphoma		**-**	

1.2.6.1 Syndecan-1 roles in proliferation and apoptosis

Syndecan-1 regulates tumor cell proliferation and survival through different mechanisms which could be tissue type dependent. Several signaling pathways have been associated with the role of syndecan-1 as a co-receptor. Syndecan-1 acts in an HS-dependent fashion as a co-receptor for Wnt signaling and syndecan-1 null mice were resistant against Wnt-1 induced mammary tumorigenesis [168].

Hepatocyte growth factor (HGF) signaling is conducted through its receptor, Met, which is important in cancer progression [169, 170]. In myeloma, HGF can bind HS chains of syndecan-1 [171]. This interaction enhances Met signaling and further PI3 kinase-protein kinase B and Ras-MAP kinase pathways activation [172]. Furthermore, in myeloma cells heparanase induces syndecan-1 shedding via MMP-9 upregulation [173] and also it increases HGF expression [174]. Shed syndecan-1 also augments this effect with binding to HGF and presenting it to osteoblasts. This results in inhibition of bone formation and promotion of bone resorption [175].

In many tumor types, the role of shed syndecan-1 in the stroma depends on the cell type and might conflict the role of cell bound-syndecan-1. Stromal syndecan-1 enhances proliferation of breast epithelial cell [176], and in ovarian [163], oral [177] and gastric carcinomas [178], it is correlated to poor outcome. On the other hand, the stromal immunoreactivity to syndecan-1 in basal cell carcinoma is negatively correlated to aggressiveness [179].

In tumor stroma, syndecan-1 shedding delivers growth factors to tumor cells and help to maintain the growth and survival of tumor cells. This is supported by data showing that ectodomain shedding of syndecan-1 from stromal fibroblasts stimulated tumor cell growth through the activation of FGF2 signaling [180]. Also, syndecan-1 overexpression in fibroblasts enhances cancer cell proliferation in mammary tumor cells [181]. Taken together, stromal syndecan-1 could store and present heparin-binding growth factors such as FGFs, HGF, and EGFs to cancer cells and enhance their proliferation [98].

Syndecan-1 could also regulate tumor cell apoptosis in dual fashion in different cell types. For instance, in myeloma cells syndecan-1 silencing induced apoptosis [182], which might be explained by reduced levels of cell surface syndecan-1 as co-receptors for growth factor signaling. However, the opposite effect has been observed in MCF-7 breast cancer cells, where the addition of human recombinant syndecan-1 ectodomain increased apoptosis [183].

It is not clear yet how exactly the cell surface and shed syndecan-1 can modulate growth and apoptosis in various cancer cells. As tumor cells can vary in terms of their requirements for growth factors, this might partly explain the differences among tissues. In addition, tumor microenvironment is unique for each tumor, and syndecan-1 HS chains could be different to either enhance or inhibit tumor cell growth or apoptosis [184].

Furthermore, HS chains play important roles as being inhibitory or stimulatory factors for a ligand activity. The activity curve for HS chains binding to their ligands might be bell-shaped, meaning that at either sides of the optimal HS concentration, ligand activity could be low or high, which could explain the opposing roles of syndecan-1 in tumor cell proliferation and apoptosis [98].

1.2.6.2 The role of syndecan-1 in angiogenesis

Syndecan-1 is able to bind to pro-angiogenic factors as VEGF and FGF-2 and presents them to their corresponding receptors on endothelial cells and enhance endothelial cells growth and sprouting. Shed syndecan-1 plays important role here in tumor angiogenesis and metastasis by increasing the local concentration of angiogenic factors to promote angiogenesis [98].

In myeloma, heparanase mediated shedding of syndecan-1 promoted angiogenesis [185]. In addition, heparanase upregulated VEGF and HGF in myeloma cells, increasing their presentation through shed syndecan-1 to endothelial cells [173, 185]. It has been shown that heparanase promotes angiogenesis through generation of HS chains [186]. However, intact syndecan-1 ectodomain is also crucial for angiogenesis, as addition of intact syndecan-1 ectodomain to rat aortas promoted angiogenesis [187].

Syndecan-1 ectodomain can bind to $\alpha_v\beta3$ and $\alpha_v\beta5$ integrins and form a ternary complex with insulin-like growth factor-1 receptor. This complex activates integrins, which are necessary for pro-angiogenic functions. The short synthetic peptide that mimics this integrin attachment sequence on syndecan-1 ectodomain (synstatin) inhibits syndecan-1 interactions with integrins and forming the ternary complex, and in myeloma blocks endothelial cell invasion and tumor growth *in vivo* [185, 188-190].

Syndecan-1 expression in stromal fibroblasts is increased in several carcinomas, such as breast cancer [181], where it correlated with larger vessel areas and high microvessel density (MVD) [187].

1.2.6.3 The role of syndecan-1 in migration, wound healing and metastasis

Membrane-bound syndecan-1 acts as adhesion molecule and increases cell adhesion to ECM, and hampers cell migration. This has been seen in squamous cell carcinoma cells where cell surface syndecan-1 loss reduced cell adhesion to collagen, but promoted cell migration and invasion [191].

Integrins are important receptors in cell adhesion and migration. Syndecan-1 can interact with $\alpha_v\beta3$ and $\alpha_v\beta5$ integrins and activate their signaling via the engagement of α_v ligands such as fibronectin (FN), laminins, vitronectin (VN), thrombospondin, and collagens [188]. In addition, syndecan-1 and integrins can link ECM to cytoskeleton and stabilize the focal adhesions, which causes limited cell migration and more adhesion [98].

Furthermore, data suggest that membrane-bound syndecan-1 and shed syndecan-1 may play different roles at different stages of cancer progression. In MCF-7 breast cancer cells, cell surface syndecan-1 inhibited the invasion, while shed syndecan-1 promoted invasion of cells into Matrigel [192]. Likewise, cell surface syndecan-1 hampered migration of fibrosarcoma cells, where syndecan-1 ectodomains stimulated cell migration [124]. MMP inhibitors increased syndecan-1 expression on the cell surface and actin stress fibers were formed and caused cell migration inhibition [124].

During wound healing, expression of MMPs and heparanase and consequently bioactive shed syndecan-1 are increased. Syndecan-1 null mice are viable, but show retarded wound healing in both skin and cornea, due to slow keratinocyte migration. It has been proposed that the

wound healing role of syndecan-1 is conducted by promoting cell migration, generating chemokine gradients, and modifying cell signaling [102, 124, 193].

Taken together, data suggest that loss of cell surface syndecan-1, which is seen in many types of carcinoma could reduce cell adhesion and enhance a more migratory and invasive capacity in cancer cells.

1.2.6.4 Syndecan-1 in mesothelioma

Cell surface syndecan-1 has been reported to be essential for maintaining normal epithelial cell morphology and anchorage-dependent growth, as loss of this PG on epithelial cells leads to anchorage independent growth, and cells can invade and migrate through collagen gels, as mesenchymal cells do [154]. In addition, reduced syndecan-1 expression has been correlated to less differentiation and malignant transformation of cells [194].

Generally, cell-bound expression of syndecan-1 in mesothelioma is relatively lower than epithelial malignancies. In MM, expression of syndecan-1 could be used as a differentiation marker, therefore associating with better prognosis. Epithelioid subtype expresses higher levels of syndecan-1, while sarcomatoid subtype which is less differentiated demonstrates low or negative syndecan-1 expression [157, 195].

Syndecan-1 overexpression on mesothelioma cells hampers tumor growth [99] and migration, while enhances cell adhesion [196]. These functional changes are along with morphological changes towards epithelioid direction [99], however syndecan-1 downregulation leads to sarcomatoid phenotype, another indication of EMT transition and worse prognosis when syndecan-1 is less on these cells [195].

MM cells also express syndecan-2 and -4, which are less expressed in carcinomas [195]. Therefore, the ratio of syndecan-1 to -2 has been proposed as differentiating biomarkers of MM from metastatic adenocarcinoma [197, 198].

2 AIMS

The general aim of this thesis is to investigate the relation between molecular structure and function of syndecan-1 in MPM. This knowledge could be helpful in facilitating early diagnosis by using syndecan-1 as diagnostic or prognostic factor and understanding modulations of various cellular signaling pathways by this potential biomarker.

Specific aims

Paper I: To identify genes and pathways affected by syndecan-1 in mesothelioma

Paper II: To assess the diagnostic and prognostic value of soluble syndecan-1 in pleural malignancies

Paper III: To investigate syndecan-1 impact on heparan sulfate structure and sulfation pattern of proteoglycans in mesothelioma

Paper IV: To explore the role of syndecan-1 in angiogenesis of mesothelioma

3 REMARKS ON METHODOLOGY

3.1 CELL LINES

Cell lines that were used in this thesis included MPM cell line STAV-AB (papers I, III and IV) and human umbilical vein endothelial cells (HUVECs; paper IV). The STAV-AB cell line, once established from a pleural effusion, grows with epithelioid phenotype and has low endogenous expression of syndecan-1 [195]. The STAV-AB cells were also transfected with a vector containing the human full-length syndecan-1 gene [99]. The level of syndecan-1 overexpression was constantly ensured before experiments using fluorescence activated cell sorting. Human umbilical vein endothelial cells (HUVECs) were purchased from ATCC.

3.2 PATIENT MATERIALS

Pleural effusions and/or serum samples from patients (paper II, III and IV) had been collected at the Department of Pathology and Cytology, Karolinska University Hospital in Huddinge, Sweden and at the Medical Faculty of Eskisehir, Department of Chest Diseases, Turkey. All samples were from chemotherapy-naïve patients. Biomarkers synthesized in the pleura are less affected by biological elimination in the restricted pleural cavity compared to when they have entered the circulation. Measurements are thus more sensitive when performed on pleural effusions compared to serum.

3.3 IMMUNE BASED ASSAYS

3.3.1 Enzyme linked immuno-sorbent assay (ELISA)

Sandwich ELISA was used in papers II, III, and IV to measure shed syndecan-1, VEGF and sulfatase-1 levels, using commercial kits. This technique is rather sensitive and provides semi-high throughput methods to measure specific antigens.

3.3.2 Proteome Profiler Array

This method was used in papers I, III, and IV. In paper I, expression of several proteins was measured to validate microarray results. In paper III receptor tyrosin kinase activity, and in paper IV angiogenesis related proteins were measured. Performing multiplex antibody array to detect multiple antigens is cost and time-effective. However, the chemiluminescence used in these assays, caused high inter-experimental variability in signal intensity causing higher standard deviations that could influence the detection of more delicate changes.

3.3.3 Fluorescence Activated Cell Sorting (FACS)

This method was generally used to validate syndecan-1 overexpression (paper I, III and IV) and to measure apoptosis (paper I), cell cycle (paper I and III) and the expression of variously sulfated regions of HS and different components of intracellular pathways (paper III). The technique allows the characterization of multiple parameters of single cells, physical properties such as size and granularity together with specific fluorescent antibody labels.

3.3.4 Immunocytochemistry

Cell-bound syndecan-1 (paper II), and expression of ERK1/2 and pERK1/2 (paper III) were measured using fluorescent immunocytochemistry, which allows the binding of antibody to the antigen. The evaluation necessitates the use of positive and negative controls.

3.4 TRANSCRIPTOMICS AND QUANTITATIVE PCR

Microarray analysis on syndecan-1 overexpressing and silenced cells was used to identify their global gene expression (paper I and III), using Affymetrix GeneChip® Human Gene 1.0 ST microarray. Gene ontology (GO) annotations were used to correlate genes with biological functions.

Differentially expressed genes (DE) in the experiment are called altered gene sets (AGS) and genes that were previously known from the literature to be related to a function are called functional gene sets (FGS). To define the transcription profiles of affected genes three different approaches were used. First, based on Gene Ontology (GO), gene set enrichment analysis (GSEA) was used, which is a method using the maximum overlap of specific function between AGS and FGS. This analysis, considers DE genes that are linked to any FGS disregarding any known functional links within the analyzed AGS.

Moreover, two different methods of gene network analysis were applied: ingenuity pathway analysis (IPA) and a novel method of network enrichment analysis (NEA). IPA performs GSEA on network modules of DE genes. IPA finds associations between AGS and known pathways or FGS, but this analysis considers the networks. Networks are modules of genes which are tightly connected. Thus, IPA considers only those DE genes that can be grouped in a network, by which errors might occur.

The other network-based method was network enrichment analysis (NEA) that finds network connectivity between AGS and FGS. This method is a combination of conventional GSEA and IPA considering the links between genes of AGS and FGS in the global network. NEA finds over/under-representation of the neighboring FGS in the gene network rather than in the AGS. NEA has a higher statistical power than GSEA, since the potential connections between gene groups are expanded from listed genes to their network neighbors [199]. All the three methods of GSEA, IPA and NEA use known links from literature searches, therefore there is always the literature bias existing and the yet unpublished possible interactions are not taken into consideration.

Sets of DE genes obtained from microarray were validated on mRNA level using quantitative real time polymerase chain reaction (qRT-PCR), which was performed with Platinum SybrGreen qPCR SuperMix-UDG kit. The quantity of DE genes was normalized to GAPDH as reference gene and to the corresponding controls.

3.5 ROC PLOT ANALYSES

The performance of syndecan-1 as a diagnostic marker for malignancy was evaluated by receiver operating characteristic plot (ROC plot). This test plots the true positive rate (sensitivity) versus the false positive rate (1-specificity) at varying cut-off values and the usefulness of the marker analysis is reflected by the area under curve (AUC). An AUC value of 1.0 indicates a perfect test (100% sensitivity and 100% specificity) while AUC of 0.5 indicates no diagnostic utility

3.6 GENE SILENCING

Transient silencing of syndecan-1 (paper I) and MMP-7 (paper IV) were performed using siRNA constructs for the corresponding gene. Scrambled siRNA sequences were used as negative controls. Silencing was validated on mRNA and/or protein level using qPCR and/or FACS. The advantage of siRNA silencing is that the effect is obtained in a few days.

3.7 ANALYSIS OF HS SULFATION PATTERNS

The fine structure of HS chains was studied by High Performance Liquid Chromatography (HPLC) as the composition of disaccharides obtained by heparinase digestion (paper III). Based on differences in binding constants to a cation, the variously sulfated compounds could be separated under reversed phase conditions (ion pair reversed phase). Obtained results were also verified by High Performance Capillary Electrophoresis (HPCE), using a silica capillary and reversed polarity. The latter needs considerably less material, but also requires further purification of the sample to be analyzed. The two methods verified each other, although HPLC gives more detailed information of less abundant sulfation pattern.

3.8 FUNCTIONAL ASSAYS

3.8.1 Proliferation assay

The effect of syndecan-1 silencing on the proliferation of mesothelioma cells (paper I) and the effect of conditioned media from syndecan-1 overexpressing cells on HUVEC cell proliferation (paper IV) were evaluated using the WST-1 assay. The assay is a quantification of mitochondrial activity and reflects the number of viable cells present. A factor to consider is that any change in metabolic activity due to treatments also may affect the results.

3.8.2 Cell cycle distribution

The cell cycle distribution of mesothelioma cells was studied by flow cytometry following permeabilization of cell membranes by ethanol fixation and propidium iodide (PI) staining. The analysis defines the cell cycle progressions from G0/G1, G2/M and S-phase. The effect of syndecan-1 silencing (paper I), of syndecan-1 overexpression (paper III) and of exposure of these cells to sulfatase-1 (paper III) was monitored in this way.

3.8.3 Apoptosis assay

The effect of syndecan-1 silencing on apoptosis was assessed using PI and FITC conjugated Annexin-V (paper I). During early apoptosis the cell membrane flips, exposing phosphatidylserine on the outside of the membrane, allowing the Annexin-V to bind. As the apoptosis progresses the cell membrane degrades, allowing PI to enter the cells, where it intercalates with the nuclear DNA. This progression is different from necrosis, where the cell membranes deteriorate, leaving PI labelled nuclei with no measurable increase in Annexin-V staining.

3.8.4 Chemtoaxis assay

The effect of conditioned media from syndecan-1 overexpressing cells on HUVEC cell chemotactic migration was measured using Transwell plates (paper IV). This method evaluates directional cell migratory capacity. HUVEC cells were seeded in the upper chamber and allowed to migrate downward through porous membrane towards the conditioned media. Percentage of covered area by migrated cells was measured with image processing software after membranes were stained with crystal violet.

3.8.5 Wound healing assay

The effect of conditioned media from syndecan-1 overexpressing cells on HUVEC cells migration was also measured using the Cytoselect wound healing assay (paper IV). HUVEC cells were seeded confluently and the wound was made using inserts in the wells. The cells were treated with conditioned media from syndecan-1 overexpressing cells, measuring the ability of HUVEC cells to close the wound area with image processing software. This method measures not only cell migration but also cell proliferation.

3.8.6 Tube formation assay

To investigate the ability of tubulogenesis of HUVEC cells due to conditioned media from syndecan-1 overexpressing cells, tube formation assay was used (paper IV). The HUVEC cells were seeded on extracellular matrix gel and incubated with conditioned media from syndecan-1 overexpressing cells. The features of tube formation were measured using image processing software. For reproducibility purposes care must be taken that the experiments are made with the commercial support matrices from the same batch.

4 RESULTS AND DISCUSSION

4.1 PAPER I: Novel genes and pathways modulated by syndecan-1: implications for the proliferation and cell-cycle regulation of malignant mesothelioma cells

Previous studies show that overexpression of syndecan-1 in MM inhibits tumor growth [99] and migration [196], along with epithelioid differentiation [99]. We also know that syndecan-1 presence on mesothelioma cells correlates with better prognosis [157]. Therefore, in this paper we mapped the molecular changes related to syndecan-1 overexpression and silencing on the genes and pathways regulated by this heparan sulfate proteoglycan in a MPM cell line. To perform this, microarray analyses were used following syndecan-1 overexpression and silencing on MM cells with combining three different bioinformatics approaches of GSEA, IPA and NEA. Transcriptomic data were validated using real time-PCR and/or proteome profiler array. Moreover, functional assays on cell proliferation, cell cycle and apoptosis were performed after syndecan-1 silencing.

Following syndecan-1 modulation either with overexpression or silencing, transcriptomic analysis demonstrated a remarkable effect on the global gene expression profile. Using a threshold of one and a half times up- or downregulation compared to controls, the number of differentially expressed genes (DE genes), were 2,389 and 103, after overexpression and silencing respectively. There were fourteen genes in common, which were affected by both overexpression and silencing of syndecan-1 (Table 1 in paper I). The lower number of DE genes by syndecan-1 silencing could be explained by the initially low levels of endogenous syndecan-1 in MPM cells, which makes the silencing less pronounced.

Functional characterization of DE genes regulated by syndecan-1 overexpression showed that these genes were involved in cell adhesion, proliferation, cell cycle progression, cell motility and migration, angiogenesis and ECM organization (Figure 4 in paper I). Furthermore, expression of cytokines (Table S4 in paper I) and TGFβ family members and their receptors and growth factors and their receptors including EGF, VEGF, PDGF and FGF were affected in syndecan-1 overexpressing cells. Since one mesothelioma cell line was used, cell line specificity of the obtained results cannot be ruled out. Downstream MAPK, JAK-STAT and TGFβ signaling pathway members were also modulated. The highly affected genes and signaling pathways affected by syndecan-1 overexpression based on KEGG pathways (depicted by Figure 5 in paper I), showed that the outcome of these alteration affects cell proliferation, differentiation and cell cycle.

NEA analysis of syndecan-1 overexpressing and silenced cells revealed that all the analysed cell cycle pathways were enriched after syndecan-1 was silenced and nearly all of them were depleted after syndecan-1 was overexpressed. This highlights the role of syndecan-1 on cell proliferation (Figure 9B in paper I).

Functional assays revealed that syndecan-1 silencing hampers cell proliferation, a finding which was seen before for syndecan-1 overexpression. However, cell cycle distribution showed a different mechanism for the two settings: due to syndecan-1 silencing cells were mostly accumulated in G0/G1 phase and less cells were in G2/M, while the effect of

syndecan-1 overexpression was more a prolonged S phase [99]. Apoptosis was not affected by syndecan-1 silencing. We hypothesized that the effect of syndecan might have a bell-shaped curve, meaning that in order to have maximum cell proliferation an optimal level of syndecan-1 is needed. Below or above this level of syndecan-1, cell proliferation is inhibited. The mechanistic effect of syndecan-1 overexpression on proliferation could partly be explained by the findings that TGFβ2 was downregulated. In tumorogenesis TGFβ may act more as tumor promoter, as in mesothelioma the reduction of TGFβ inhibits tumor growth [200]. On the other hand sulfatase-1 was down regulated upon syndecan-1 overexpression. This can be debating as sulfatase-1 decreases the growth factor binding affinity of heparan sulfate proteoglycans with removal of 6-O-sulfation of heparan sulfate chains [118]. The change in sulfatase-1 was together with other enzymes involved in heparan sulfate sulfation patterning (SULT1B1 and SULT1E1), which were significantly downregulated upon syndecan-1 overexpression. All these disclose a possibility of GAG change and consequently growth factor binding and intracellular signaling modifications in these cells.

Altogether, the data in paper I indicate that syndecan-1 regulates high number of genes and pathways profoundly affecting cell proliferation in mesothelioma cells.

4.2 PAPER II: Diagnostic and prognostic value of soluble syndecan-1 in pleural malignancies

Pleural effusion is usually the first available diagnostic material in pleural malignancies. However, differentiation between benign and malignant pleural effusions is still challenging. Measuring soluble biomarkers in pleural effusions could be an aid in diagnosis. Syndecan-1 can be shed into biological fluids and might have conflicting roles as membrane-bound in cancer. Further, syndecan-1 could be either tumor suppressor or promoter in different cancer types [201].

In paper II, we explored the differential diagnostic and prognostic value of soluble syndecan-1 in patients' serum and pleural effusions from benign and malignant effusions including: lung cancer, breast cancer, ovarian and fallopian cancers, other malignancies, cancer of unknown primary, and MPM. Osteopontin is an established malignancy marker, which was used as a control.

In pleural effusions, both syndecan-1 and osteopontin levels were higher in malignancies than benign conditions and the difference was even more pronounced for syndecan-1. However, this discriminatory performance was not seen in sera either for soluble syndecan-1 or osteopontin. Among the pleural malignancies, in all malignancies except for ovarian and fallopian carcinoma, syndecan-1 expression was higher in pleural effusions compared to benign disease. While, there was no significant difference in syndecan-1 in serum of these patients (Figure 1 and 2 in paper II).

To investigate the diagnostic value of soluble syndecan-1, ROC curve was created. Syndecan-1 true positive rate (sensitivity) was plotted over its false positive rate (100% - specificity), and the area under this curve (AUC) will give us a measure of the syndecan-1's overall accuracy. Soluble syndecan-1 in pleural effusion showed diagnostic value for malignancy from the benign cases, while in sera, syndecan-1 showed poor prediction of a

malignant disease (Figure 3 in paper II). Both sensitivity and specificity of syndecan-1 in pleural effusions was slightly higher than osteopontin (Table 2 in paper II).

To study the prognostic value of soluble syndecan-1 Kaplan-Meier analysis were used. In pleural effusion of patients with pleural metastases, lower syndecan-1 levels than 235.1 ng/mL resulted in longer survivals compared to patients with higher syndecan-1. The same results were seen with effusion osteopontin levels with a cut off of 6143 ng/mL. Stratifying MM patients in "low" and "high" syndecan-1 level in pleural effusions using a cutoff of 100.2 ng/mL demonstrated longer survival times of low syndecan-1 levels compared to high levels. Similar results were seen for effusion osteopontin levels with a cut off of 1630 ng/mL (Figure 4 in paper II).

The difference seen in pleural effusions, and not in sera can be explained by less interference by liver metabolism or elimination by the kidneys and also the lower contribution of other body fluids into pleural effusions, these effusions are known to have higher diagnostic accuracy than serum for several MM biomarkers [38, 202]

Syndecan-1 ectodomain can act as soluble receptor for biological effectors such as growth factors and chemokines, therefore syndecan-1 shedding is important in regulating signaling pathways and possibly cancer progression and affecting patient's survival [108, 115]. However, it is noteworthy that increased level of soluble syndecan-1 could be due to tumor burden as well and in this way affecting survivals.

Taken together, this study shows that effusion content of syndecan-1 has diagnostic and prognostic values and combining syndecan-1 with other biomarkers to diagnose pleural malignancies could be advantageous. However, the use of syndecan-1 measurement in serum is limited for such performance.

4.3 PAPER III: Syndecan-1 alters heparan sulfate composition and signaling pathways in malignant mesothelioma

Syndecan-1 binds a wide range of heparin-binding growth factors through its polysaccharide chains and modulates the availability, stability, and/or activity of these binding partners consequently affecting downstream signaling pathways [203, 204]. Therefore, studying HS chain modifications could be crucial in different tumor types. In paper I, we reported that at transcriptomic levels, several enzymes involved in HS sulfation modifications were affected by overexpressing syndecan-1 in mesothelioma. One of the highly-affected enzymes derived from that study, was sulfatase-1 which removes 6-O-sulfates from HS chains. Therefore, in paper III we investigated syndecan-1 overexpression effect on HS chain composition and affected signaling pathway members in mesothelioma. In addition, the correlation between soluble syndecan-1 and sulfatase-1 in pleural effusions of cancer patients was studied.

To explore the effect of syndecan-1, the expression of important enzymes involved HS biosynthetic and modifying machinery was studied both at RNA and protein levels. The most highly affected enzyme at RNA level was sulfatase-1. In addition, expression of EXT1, NDST1, HS2ST1, HS6ST1, SULF1 and the sulfate donor synthase PAPSS1 were affected by syndecan-1 overexpression (Figure 1a in paper III). Since both EXT1 and NDST1 were

transcriptionally downregulated, we postulated a negative feed-back in the syndecan-1 overexpressing cells which leads to slower heparin sulfate biosynthesis.

Using antibodies against various sulfated saccharide motifs revealed that syndecan-1 overexpressing cells contained HS chains with higher contents of 6-O- and N-sulfation (Figure 2 in paper III). The chromatographical analysis showed that syndecan-1 overexpression decreased total amounts of HS in both medium and cell samples (Figure 4a, b in paper III). This could be due to slower HS biosynthetic machinery as explained above. Furthermore, we know from previous studies that overexpression of syndecan-1 could downregulate other HSPGs in a compensatory way [99, 205].

Despite decreased amount of HS chains due to syndecan-1 overexpression, the overall sulfation of these chains was higher, mainly in N- and 6-O-sulfation (Table 4 in paper III), and more in details 6-O-sulfated disaccharides were affected (Figure 4c in paper III). This is in line with low expression of sulfatase-1 in these cells.

Studying the downstream effect of HS chains modification due to syndecan-1, on mitogen-activated protein kinase (MAPK)-related components, we showed using two independent methods that EGFR was higher in syndecan-1 overexpressing cells compared to controls, but the difference did not reach statistical significance. Moreover, phosphorylation of protein kinase WNK1 which is known to be apoptosis inhibitor, and Akt 1/2/3, an important factor in the G1/S transition checkpoint were decreased in syndecan-1 overexpressing cells (Figure 6 and 8 in paper III). Elevated levels of both total and phospho-ERK1/2, in cells overexpressing syndecan-1 was shown by both flow cytometry analysis and immunocytochemistry (Figure 7 in paper III). The activity of transcription factor c-Jun which is necessary for the expression of cyclin D1 and G1/S progression was inhibited by syndecan-1 overexpression. The expression of ETS-1 transcription factor was increased, but its activity was not affected (Figure 9 and 10 in paper III). The elevated ETS1 levels with no changes in activity can lead to the conclusion that ETS-1 activation was inhibited via a signaling pathway independent of ERK1/2. All these inhibition of Akt 1/2/3, c-Jun, and ETS1 ultimately resulted in cell cycle arrest due to syndecan-1 overexpression (Figure 11 in paper III).

Sulfatase-1 expression was low in malignant pleural effusions indicating a potential diagnostic role for this enzyme in pleural malignancies. However, no prognostic effect in pleural malignancies was found for sulfatase-1. In addition, there was a fair inversed but not significant correlation between soluble syndecan-1 and sulfatase-1 in pleural effusion of mesothelioma patients (Figure 12 in paper III). These finding together mirror the results from paper II, where we found high levels of syndecan-1 in pleural malignancies.

4.4 PAPER IV: Syndecan-1 overexpressing mesothelioma cells inhibit proliferation, migration and tube formation of endothelial cells

Angiogenesis is crucial in tumor development and mesothelioma progression. Poor survival in mesothelioma patients is associated with high microvascular density and angiogenesis-related proteins have been implicated in adverse prognosis [78, 79, 206]. However, anti-angiogenesis treatments have shown modest results in mesothelioma patients. Syndecan-1 can affect angiogenesis by acting as a co-receptor for extracellular ligands such as VEGF and FGF [98, 207] and interacting with $\alpha_v\beta3$ and $\alpha_v\beta5$ and regulating angiogenesis by activation

of these integrins and insulin-like growth factor-1receptor (IGF-1R) forming a ternary receptor complex. This complex is present mainly on tumor cells and activated endothelial cells during angiogenesis [208]. Moreover, syndecan-1 ectodomain can get shed by sheddases and this process generates soluble syndecan-1 receptors, which can actively participate in angiogenesis.

In this paper, we investigated the role of syndecan-1 in angiogenesis. First shed syndecan-1 level was confirmed in the conditioned media from syndecan-1 overexpressing mesothelioma cells. The angiogenesis-related proteins of conditioned medium from syndecan-1 overexpressing cells were determined. Several angiogenesis-related proteins were altered including pro-angiogenic Angiopoietin-1, fibroblast growth factor-4 (FGF-4), hepatocyte growth factor (HGF), neuroglin-1 beta-1(NRG1-β1), and anti-angiogenic protein tissue inhibitor of metalloproteinases-1 (TIMP-1) and pro/anti-angiogenic proteins such as thrombospondin-1 (TSP-1) and transforming growth factor beta-1 (TGF-β1) (Figure1 in paper IV). Conditioned medium from syndecan-1 overexpressing mesothelioma cells inhibited endothelial cell proliferation, migration and tubulogenesis (Figure 2, 3 and 5 in paper IV). Migratory capacity of endothelial cells in presence of syndecan-1 overexpressing cell conditioned media was tested via both wound healing and along with chemotactic gradient. Endothelial cell migration was inhibited in wound healing setting but chemotactic migration of endothelial cells was not changed in presence of conditioned medium from syndecan-1 overexpressing cells.

This inhibition of angiogenic characteristic of endothelial cells show that shed syndecan-1 and angiogenesis-related proteins in the conditioned media from syndecan-1 overexpressing cells can affect angiogenesis.

To explore the role of shed syndecan-1 per se on the tube formation of endothelial cells, we silenced MMP-7 expression in mesothelioma cells. MMP7 has been shown to be the main cause of syndecan-1 shedding from lung epithelium in response to injury [123]. As an effect of MMP-7 silencing on syndecan-1 shedding we observed that syndecan-1 was decreased by nearly 30% in the MMP-7 silenced supernatant compared to scrambled control (Supplementary Fig. 2B in paper IV). Although, MMP-7 was not fully silenced, the activity of other syndecan-1 sheddases might still be present. The partial inhibition of syndecan-1 shedding restored the tube formation inhibition by syndecan-1. Thus, it can be concluded that presence of shed syndecan-1 seems to be crucial for angiogenesis inhibition (Fig. 5C and D, Fig.6, and Supplementary Fig.1 in paper IV).

Yes-associated protein (YAP) is an effector of Hippo pathway and gets normally degraded in the cytoplasm. In MM, NF2 inactivation results in nuclear localization of the YAP and its activation [209]. The nuclear localization of YAP promotes angiogenesis, proliferation and migration in cancer [210]. We showed that endothelial cells manifest slightly more cytoplasmic and less nuclear YAP in presence of conditioned media from syndecan-1 overexpressing cells compared to control. This is in line with hampered endothelial migration by shed syndecan-1 that described above.

VEGF is one prominent angiogenic factor, which is previously proposed as a biomarker of tumor progression and angiogenesis in malignant effusions. Study from pleural effusions

from mesothelioma patients showed that shed syndecan-1 is positively correlated with VEGF. In addition, mesothelioma patients with higher levels of VEGF in pleural effusions displayed shorter survivals (Figure 8 in paper IV). Results from paper II show that mesothelioma patients have higher levels of shed syndecan-1 in pleural effusions compared to benign cases. Taken together these data suggest that combining VEGF and shed syndecan-1 in pleural effusions could be helpful prognostic tool in mesothelioma patients.

5 GENERAL CONCLUSION

5.1 MAJOR FINDINGS

Paper I: Genes and pathways modulated by syndecan-1 in mesothelioma

- Syndecan-1 silencing and overexpression in mesothelioma cells greatly affect cellular signaling.
- Syndecan-1 is functionally linked to cell proliferation.
- Syndecan-1 overexpression shows larger impact on differentially expressed genes than syndecan-1 silencing.
- Syndecan-1 overexpression affects the expression of growth factors and their cognate receptos, TGFβ signaling pathway-related members, and causes GAG modifications.

Paper II: Syndecan-1 and diagnostic/prognostic value in pleural malignancies

- Shed syndecan-1 in malignant pleural effusions is higher than benign conditions, therefore it carries diagnostic value.
- Syndecan-1 is a better diagnostic/prognostic marker in pleural effusion than in serum.
- Mesothelioma patients with higher syndecan-1 levels in pleural effusions show shorter survival time.

Paper III: Syndecan-1 alters heparan sulfate chains and signaling pathways in mesothelioma

- Syndecan-1 overexpression affects HS biosynthetic/modifying enzymes mainly downregulating sulfatase-1.
- HS content was reduced due to syndecan-1 overexpression, but overall sulfation was increased.
- As an effect of these changes in HS structure, syndecan-1 modulates PI3K and MAPK signaling pathways in mesothelioma cells, resulting in deregulation of cell cycle progression.
- Pleural malignancies demonstrate lower sulfatase-1 in pleural effusions compared to benign conditions, and sulfatase-1 level inversely correlates with soluble syndecan-1 level.

Paper IV: Syndecan-1 role in angiogenesis of mesothelioma

- Syndecan-1 overexpression on mesothelioma cells change angiogenicity by affecting the growth factors gradient and inhibiting endothelial cells proliferation, migration and tube formation.
- Mesothelioma patients with higher VEGF level in pleural effusions had shorter survivals.
- VEGF level in pleural effusions from mesothelioma patients is positively correlated with soluble syndecan-1.
- Combining VEGF with other biomarkers in pleural effusion could give a better prognostic evaluation in mesothelioma patients.

6 FUTURE PERSPECTIVE

This thesis focuses on the new functional and clinical roles of syndecan-1 and related genes and pathways in MM. Although the primary hypotheses were addressed, there are findings which merit further investigation.

In general, it is worthy to investigate the role of silenced syndecan-1 as complementary approach to overexpression, where the study has been conducted via syndecan-1 upregulation only. In addition, using different mesothelioma cell lines could highlight the general applicability of our findings.

Differentially expressed genes due to syndecan-1 modulation represent functional categories such as angiogenesis, adhesion, migration and proliferation which motivate future *in vivo* investigation. Moreover, syndecan-1 seems to be a powerful suppressor of TGFβ pathway in mesothelioma, which needs more mechanistic studies.

Future translational studies are needed to reveal the mechanism behind correlation between shed syndecan-1 levels and mesothelioma patients' survival. Whether metalloproteinases are involved in this high level of soluble syndecan-1 in effusions or tumor burden and malignancy affect the amount of soluble and membrane-bound syndecan-1, remained to be answered more in details. In addition, to elucidate the relationship between cell-bound and shed syndecan-1, the number of mesothelioma samples should be increased.

Prognostic role of sulfatase-1 could be evaluated with larger clinical material. Furthermore, the exact mechanism behind inhibitory role of sulfatase-1 per se on cell cycle progression remains to be disclosed.

To elucidate the role of shed syndecan-1 in angiogenesis by a more potent inhibitor of syndecan-1 shedding than the used MMP7 silencing would worth to study. This can be done via simultaneous silencing of other important enzymes in syndecan-1 shedding, or syndecan-1 immunoprecipitation from conditioned media from mesothelioma cells.

The direct effect of mesothelioma on endothelial cells seems to be interesting in the context of YAP expression. This can be performed in 3D culture to simulate better the interaction between two cell types.

7 ACKNOWLEDGEMENTS

"Your assumptions are your windows on the world. Scrub them off every once in a while, or the light won't come in."

— Isaac Asimov

Many moments of my PhD journey are passing quickly in my head, the challenging moments of confusing results, exciting moments of happy faces around me, conferences, great talks with colleagues, getting inspired by people, collaborations, taking courses, etc. I think the joy I got during these years is very meaningful. I believe that learning science has something miraculous that goes beyond the work per se, it affects your lifestyle, your way of thinking and acceptance. It truly opened my eyes before many facts that are barely found somewhere else. I am very happy about my decision to study further, though it significantly increased my gray hair!☺

There were many inspiring people that I met during my PhD, and my smart supervisor was one of the firsts I met during the interview. **Katalin Dobra**, thank you for having me as your PhD student, your valuable advice and patience during these years. You have been always present to guide me with different means to overcome obstacles that I faced during my research. I appreciate that you believed in me and gave me the possibility to test what I was eager. It was always interesting to check my email and see I have an email from you, it was sort of mysterious and at the same time joyful!☺ I will never forget your happy energetic smile and the positive dynamic atmosphere you provide.

My knowledgeable co-supervisor **Anders Hjerpe**, I am grateful to be one of your students. Thank you so much for accompanying me during my study. It has been always open-minding to talk to you. I learned a lot about the cancer biology and also being motivated, active and fresh as you are. The scientific stories that you share sometimes are always very interesting to hear.

Tünde Szatmári, my other co-supervisor and my friend, thank you for being with me both in the lab and office. Thank you for all the detailed discussions that we had, around the experiments and life. You supported me in many aspects, and I will remember them all. It was always exciting to have a mini-meeting with you and get things clarified. I loved it! I will never forget your unlimited desire for sightseeing when we were on conference and we had only half an hour off! You never get tired!☺

A special thanks to our collaborators at KI, **Andrey Alexeyenko** and **Pontus Aspenström**, in Turkey **Muzaffer Metintas** and his group, in France **Romain Vivès** and his group and in Greece **Nikos Karamanos** and his group. Our work became more meaningful with your contributions.

Thank you **Åsa-Lena Dackland** for helping me with FACS and ordering materials. It was always nice to sit beside you and talk with you in dissertation parties and fikas.

I would like to thank **Göran Andersson**, head of pathology division for all his great impacts on division to provide a happy collaborative atmosphere.

To My present/past peers in pathology division: **Joman** it was fun to collaborate with you and discuss scientific and sometimes philosophical things! **Ashish**, sharing office with you was cool, you are such an organized person. **Carl-Olof**, we got registered in the same day, it was nice to have a company during my journey. **Sulaf**, thank you for the nice discussions regarding the work and motherhood, you always give me energy with your smile. **Filip** and **Adam**, my bros in the group, I am happy that a part of my PhD overlapped yours, you were always caring and funny. **Christina** and **Anja**, it was very memorable to be with you in Greece for the conference, good old days… **Agata** and **Sougat** thanks for being responsible for journal clubs/seminars and giving me valuable advice. Agata shopping with you was fun too!☺ **Annelie, Antje, Arun, Davit, Francesco, Hanna, Laia, Lisa, Magali, Maria, Marita, Martin, Massoud, Mia, Mihaela, Raghu, Rim, Roberto, Sara, Shuvojit, Tuomas** and **Vicky** you were all very special to me and I enjoyed sharing moments with you in the lab, lunch, fika, after work, on train, etc.

Gareth Morgan, thank you for all the positive greetings, professional pictures and being my teacher in pathology course. You are one of the cheerful people I never forget.

Andrej Weintraub, Anthony Wright, Marita Ward and **Arja Kramsu**, thank you for helping me with doctoral education in department.

I would like to express my appreciation to **Karolinska Institutet** for all the facilities and fantastic programs and courses. I found it very exciting to study here and go after my interests.

Dilruba, the writing course at Solvik is one of my spectacular memories with you. You have been always caring and open to hear my stories. Thank you for being my friend.

I appreciate your friendship **Azadeh Zaferani**. You and **Jaap** were the ones who introduced me to Katalin's group. I am still very happy with your recommendation.☺

Maryam Salehi thank you for beautifully depicting the idea for the cover page. It became better than my imagination. It is always good to have an artist friend, especially one from the childhood.

My other good friends, **Maryam, Raoul, Sepideh, Behzad, Leila, Babak, Sara, Zahra, Farzaneh, Shohreh, Aboozar, Naghmeh, Omid, Ahmed, Sayyeda, Shanie, Ali, Eva**, **Mahparvar, Elham, Sara, Fatemeh, Farinaz, Najva, Zohre** and **Vida** thank you so much for the great memories and quality time we had together. I am very lucky to have you in my life.

My brilliant caring mom and dad, I dedicate this book to you maybe I can somehow express my gratitude for all your effort for raising me. Thank you for all the unconditional support and love you give me every single second of my life. You have been helping me in challenging situations. My mom **Behjat** and my dad **Hassan**, together you are the full package of heart and wisdom. When Golrokh and I came to Sweden, you indeed felt alone with no other child around, but you never ever complained. Instead, you encouraged us to continue our study and be helpful for the world, regardless of the borders. You showed me to go after my desire whatever it is and to not worry about the outcome too much. You said ‘’

you can do it, just if you want!'' That was a big lesson. You are my parenthood templates and I hope I can be as good as you!

My smart sister **Golrokh**, my kind brother in law **Maziar** and my adorable niece **Golsa**, you always motivate me with love and care. Thank you for being with me in Sweden. I am very lucky to have you by my side. It has always been heartwarming to share feelings with you. Golsa adds more to this, and I just can't describe how much I love my happy cutie pie ☺

My amazing grandma, **maman Bita**, you are my role model. Being brave, strong, independent and kind are just examples that I learn from you on daily basis and try to implement them in my work and personal life. Thank you for your warm words when I talk to you and the fantastic food you make when we are at your home.

My dear **khale Bita** and **khale Badri**, you have been like my sisters through my whole life. The Maths and Physics lessons I got from you were starting points for me to think logically. I am very thankful for the beautiful summer days you spent on teaching me. I miss you both in my life, though knowing that you have a great life in US warms my heart.

My love **Dariush**, together we have traversed this journey. You know all the stress and happiness behind it and this have made you the best empathic life partner. Every time I look back at our path, I become more confident that we evolved together with almost the same pace. Sometimes there have been gender discrepancies though, hehe. I believe if we keep this comparable pace, we can ''live happily ever after''!☺ Thank you for being there for me whenever/ wherever I needed you. You always give me ideas to think outside the box. I am confident that our KI/KTH mutual product is very proud of you, just like his mom! All the trips with you during my PhD made me fresh and active. My in laws, thank you for your love and believing in me, I appreciate that.

My gorgeous baby, **Simon**: those experiments that we did together when you were in my tummy worked the best! Then you partly became my co-author of this book!☺ Sorry though if your name is not written, you will write your own book in the future I hope! (honestly I'm thinking you ''should''! hehe) Thank you for your understanding and no tear behind me when I need to go to work. I have to tell you that with you I am the strongest that I can be. Love you to the moon and back.

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